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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Estrogen receptor-alpha (ER) mediated inhibition of NF-κB contributes to the anti-inflammatory and protective effects of estrogen in bone, cardiovasculature, and perhaps breast cancer. Cross talk could be caused by direct or indirect association of these transcription factors, or by competition for other components of the transcriptional apparatus. In order to distinguish among these possibilities, we identified clonal variants of ER(+) MCF-7 breast cancer cells that either do (MCF-7 SI), or do not (MCF-7 SS) display ER mediated inhibition of NF-κB transcriptional activity. Transient transfection of various coactivators into the MCF-7 SS cells revealed that only CBP was able to promote an inhibitory effect of estradiol on NF-κB activity. Western Blot analysis showed that CBP protein levels were reduced in this cell line relative to the MCF-7 SI cells. Both immunofluorescent microscopy and co-immunoprecipitation showed an association between ER and NF-κB in the MCF-7SI cells. CBP also immunprecipitated with ER and NF-kB in these cells. Gel shift analysis showed that estrogen treatment had no effect on the TNF-alpha induced DNA binding capability of NF-kB, suggesting that other mechanisms must be involved. ER binding to the CH/3 domain of CBP may be crucial to this effect.

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TITLE: Estrogen Receptor Inhibition of NF-(kappa)B Activity in Breast Cancer

PRINCIPAL INVESTIGATOR: Kendall W. Nettles, B.A.

CONTRACTING ORGANIZATION:

The University of Chicago
Chicago, IL, 60637

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Estrogen receptor-alpha (ER) mediated inhibition of NF-κB contributes to the anti-inflammatory and protective effects of estrogen in bone, cardiovasculature, and perhaps breast cancer. Cross talk could be caused by direct or indirect association of these transcription factors, or by competition for other components of the transcriptional apparatus. In order to distinguish among these possibilities, we identified clonal variants of ER(+) MCF-7 breast cancer cells that either do (MCF-7 SI), or do not (MCF-7 SS) display ER mediated inhibition of NF-κB transcriptional activity. Transient transfection of various coactivators into the MCF-7 SS cells revealed that only CBP was able to promote an inhibitory effect of estradiol on NF-κB activity. Western Blot analysis showed that CBP protein levels were reduced in this cell line relative to the MCF-7 SI cells. Both immunofluorescent microscopy and co-immunoprecipitation showed an association between ER and NF-κB in the MCF-7SI cells. CBP also immunprecipitated with ER and NF-kB in these cells. Gel shift analysis showed that estrogen treatment had no effect on the TNF-alpha induced DNA binding capability of NF-kB, suggesting that other mechanisms must be involved. ER binding to the CH/3 domain of CBP may be crucial to this effect.

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Annual Summary Training Report for May 28, 2001 – May 27, 2002

Key Accomplishments (see below, and attached abstract for details)

- Screening of coactivators identified CBP as sufficient for mediating inhibition of NF-κB by ER.
- ER, NF-κB, and CBP physically associate.
- Reduction in DNA binding by NF-κB in not the mechanism of inhibition.
- The CH/3 domain of CBP binds ER, and can relieve the suppression of NF-κB transcriptional activity by ER.
- RNAi of CBP is effective in reducing CBP protein levels.

Reportable Outcomes

Abstract, 2001 Endocrine Society Annual Meeting

Training and Research Accomplishments Related to Statement of Work (Statement of Work attached as appendix)

My thesis committee made several constructive criticisms of this proposal that mirrored the reviewer's comments, namely that is was too broad in scope. They suggested that I narrow the focus of the proposal to the biochemical analysis of the mechanism, and not perform the animal studies outlined in Task 3. They also suggested identifying the relevant coactivator in Task 2 before completing Task 1, which examines the details of the interactions. Since Task 2 identified CBP as the important coactivator, it was examined in Task 1 rather than the p160 coactivators. The chromatin immunoprecipitation assay was also recommended as a more physiological measure of protein interactions. I propose replacing the current Task 3 with an examination of the CH/3 domain of CBP. This domain has been previously reported to bind PCAF. The PCAF acetyl transferase activity has also been shown to contribute to NF-κB transcriptional activity. I propose to examine if ER competes for PCAF binding to CBP in vitro, and on the NF-κB enhancer of the MCP-1 gene, using the chromatin immunoprecipitation assay. The structural basis of this interaction is also important for the development of therapeutics that promote the anti-inflammatory effects of estrogen. I propose to co-crystallize the CH/3 domain of CBP with ER. The NMR structure of this domain has been recently solved, suggesting that purification and expression are possible, and that the phases can be determined using the previously solved structures. Our laboratory has extensive experience in co-crystallization of the estrogen receptor with various peptides.

Task 1: To test the hypothesis that p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα, but not for NF-κB function.

1a. Construction of GST-ER completed.

1b. Rather than use antisense oligos, small double stranded RNA (RNAi) against CBP were transfected into Cos-7 cells. Protein expression of CBP was greatly reduced without effecting expression of NF-κB p65.

1c. GST-ER pulled down the C/H3 domain of CBP. A construct with mutation in the AF2 region was also able to elicit this association, suggesting that the interaction region lies elsewhere.

1d. The effects of CBP RNAi will be examined on NF-κB transcription during months 12-36.

1e. The Stratagene "Cytotrap" yeast two-hybrid method proved to be technically challenging for expression of ER in yeast. The fusion constructs of various domains of ER to SOS did not express in yeast. Immunoprecipitation, chromatin immunoprecipiation, and immunofluorescence have been used instead. The chromatin immunoprecipitation assay has been used to show NF-κB binds to the MCP-1 enhancer in intact cells. This assay will be used to examine the binding of CBP and ER during months 12-36.

Task 2: To test the hypothesis that coactivators binding directly to ERα and NF-κB mediate the inhibitory interactions between them.

2a. Association of ER with HSP90. My committee suggested not performing this experiment.

2b-e. The MCP-1 luciferase reporter was used to examine the effects of the p160 coactivators, CBP, p300, and PCAF on NF-κB transcriptional activity. CBP and p300 were sufficient for mediating inhibition of NF-κB by ER. Deletion mutants of CBP were examined for dominant negative effects in transient tranfection. Constructs containing only the n-terminal NF-κB interacting domain, or the CH/3 domain were effective as dominant negatives at relieving the suppression of NF-κB by ER. Larger constructs, containing both regions, were not effective as dominant negatives. Western Blot analysis of ER and p65 protein levels in cells transfected with CBP or p300 will be examined during months 12-24.

Revised Task 3: Examine the role of the CH/3 domain of CBP in the ER- mediated Inhibition of NF-κB, and the structural basis of the CH/3 CBP- ER association.

1a. Test if in vitro translated PCAF competes away CH/3 CBP binding to GST-ER (Months 12-24).

1b. Examine if ER competes for PCAF binding to the NF-κB enhancer of the MCP-1 gene using chromatin immunoprecipitation (months 12-36).

1c. Clone the CH/3 domain of CBP into a bacterial expression vector (months 12-14).

1d. Express and purify CH/3 CBP and ER (months 12-16).

1e. Cocrystallize ER and CH/3 CBP (months 16-24).

1f. Crystal structure determination (months 24-36).

APPENDIX: ABSTRACT OF WORK TO DATE

ESTROGEN RECEPTOR INHIBITS NF-KAPPA-B TRANSCRIPTIONAL ACTIVITY THROUGH PHYSICAL ASSOCIATIONS WITH CBP

Kendall W. Nettles and Geoffrey L. Greene The Ben May Institute for Cancer Research. The University of Chicago, Chicago, IL 60637

Estradiol has protective effects in the cardiovasculature and bone associated with estrogen receptor-alpha (ER) mediated inhibition of NF-kB, a key transcriptional regulator of inflammation. This anti-imflammatory property may also explain the beneficial prognosis associated with ER positivity in a subset of breast cancer patients. Cross talk could be caused by direct or indirect association of these transcription factors, or by competition for other components of the transcriptional apparatus. In order to distinguish among these possibilities, we identified clonal variants of ER(+) MCF-7 breast cancer cells that either do, or do not display ER mediated inhibition of NF-kB transcriptional activity. Transient transfection assays were performed with a luciferase reporter containing the Monocyte Chemoattractant Protein-1 (MCP-1) promoter linked to its endogenous NF-kB response element. MCF-7SI (Steroid Insensitive) cells, selected for growth in charcoal stripped serum, showed a 5-10 fold enhancement of NF-kB activity after treatment with TNF-alpha, with estradiol suppressing this activity by 40-90%. The MCF-7SS (Steroid Sensitive) cell line, selected for strong growth sensitivity to estradiol, demonstrated a similar induction of NF-kB activity, but no suppressive effect of estradiol. Since CBP is reported to interact with ER and NF-kB through distinct domains, we probed its role in this interaction. CBP mediates contacts with the basal transcription apparatus, and displays histone acetyl activity that is thought to be important for the relaxation of chromatin into a transcriptionally active conformation. CBP levels were reduced in the MCF-7SS cells, while transfection of CBP enabled a dose-dependent suppression of NF-kB transcriptional activity by estradiol in these cells. Both immunofluorescent microscopy and immunoprecipitation showed an association between ER and NF-kB in the MCF-7SI cells. CBP also immunprecipitated with ER and NF-kB in these cells. Gel shift analysis showed that estrogen treatment had no effect on the TNF-alpha induced DNA binding capability of NF-kB, suggesting that other mechanisms must be involved. Preliminary data using chromatin immunoprecipitation shows that ER localizes to the NF-kB response element in the MCP1 enhancer. Using CBP deletion mutant constructs, GST-ER was shown to associate with the CH/3 domain of in vitro translated CBP. Transfection of the CH/3 domain was also able to reverse the inhibitory effects of ER on NF-κB transcriptional activity. These data strongly support a model in which ER and NF-kB physically associate, and that recruitment of CBP is associated with transcriptional repression of NF-kB. Understanding the molecular basis of these interactions is an important step in the development of better therapeutics for treatment of breast cancer, cardiovascular disease, and osteoporosis.

The U.S. Army Medical Research Materiel Command under DAMD17-BC-000661 supported this work.

Statement of Work:

Task 1: To test the hypothesis the p160 coactivators multimerize, assemble onto each LBD of an ERα dimer, and are redundant for ERα, but not NF-κB function.

a. Develop and purify ER and GST-ER-LBD and dimerization defective GST-ER-LBD proteins, including mutagenesis of GST-ER-LBD plasmid (months 1-6).
b. Develop and characterize p160 antisense nucleotides in cell culture, including northern and western blot analysis for p160 family members. (months 1-12).
c. Perform pull-down assays. (months 1-18).
d. Perform transient transfection assays with antisense nucleotides (months 12-24).
e. Confirm protein interactions with yeast two hybrid system (months 24-36).

Task 2: To test the hypothesis that coactivators binding directly to ERα and NF-κB mediate the inhibitory interactions between them.

a. purify ER from MCF-7 extracts under conditions that allow retention of chaperone complex, followed by western blot analysis for ER, p65, and HSP90 (months 1-12).
b. Development of luciferase reporter with estrogen and NF-κB response elements (months 1-6).
c. Perform transient transfection assays with transcriptional coactivator expression plasmids (months 6-24), verifying ER and p65 protein expression by western blot.
d. Development of ASC-1 and pCAF expression plasmids (months 12-24).
e. Transient transfection of ASC-1 and pCAF, determination of NF-κB and ER DNA binding activity in cell extracts (months 24-36).

Task 3: To test the hypothesis that ERα -mediated suppression of NF-κB activity elicits a less aggressive in human breast cancer cells implanted into mice.

a. Development of retroviral contructs (months 1-8).
b. Tranduction of cell lines (MCF-7 + empty vector, MCF-7 + IKKβmutEE, MB231 + empty vector, MB231 + IKKβmutEE, ER-MB231 + empty vector, ER MB231 + IKKβmutEE), characterization of NF-κB activity and viability (months 8-12).
c. Implantation of cells into mice (months 12-18).
   Number of subjects: 12 mice/ group= A total of 72 mice.
d. Histological analysis (months 18-36).